

BEST AVAILABLE COPY

RECEIVED  
22 APR 2004

WIPO PCT

PI 1155189

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office

April 19, 2004

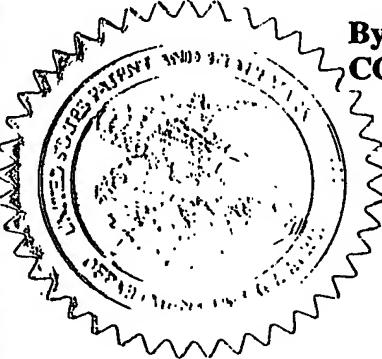
THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM  
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK  
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT  
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A  
FILING DATE.

APPLICATION NUMBER: 60/443,783

FILING DATE: January 30, 2003

RELATED PCT APPLICATION NUMBER: PCT/US04/02543

By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS

  
P. R. GRANT  
Certifying Officer

PRIORITY  
DOCUMENT  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

10687 U.S. PTO  
01/30/03

1-31-03

604-43783-013003

Approved for use through 10/31/2002. OMB 0651-0032  
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

## PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 115017985 US

10687 U.S. PTO  
01/30/03

### INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Ronald D.	Guiles	Columbia, MD

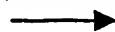
Additional inventors are being named on the \_\_\_\_\_ separately numbered sheets attached hereto

### TITLE OF THE INVENTION (500 characters max)

Novel Compounds for the Treatment of Sickle Cell Disease

Direct all correspondence to:

### CORRESPONDENCE ADDRESS

Customer Number **33,758**    
OR *Type Customer Number here*

Firm or  
Individual Name

PATENT TRADEMARK OFFICE

**33758**

Address

Address

City

State

ZIP

Country

Telephone

Fax

### ENCLOSED APPLICATION PARTS (check all that apply)

Specification Number of Pages **12**  CD(s), Number   
 Drawing(s) Number of Sheets **8**  Other (specify)   
 Application Data Sheet. See 37 CFR 1.76

### METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

Applicant claims small entity status. See 37 CFR 1.27.  
 A check or money order is enclosed to cover the filing fees  
 The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: **21-0684** **\$80.00**  
 Payment by credit card. Form PTO-2038 is attached.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

No.

Yes, the name of the U.S. Government agency and the Government contract number are: **National Science Foundation, Grant Number MCE9902244**

Respectfully submitted,

SIGNATURE 

TYPED or PRINTED NAME **David L. Marks**

TELEPHONE **410-706-1933**

Date **01/30/2003**

REGISTRATION NO.  
(if appropriate)  
Docket Number:

**37,881**

**RG-2002-002**

### USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

## COMPOUNDS FOR THE TREATMENT OF SICKLE CELL DISEASE

Field of the invention

This invention relates to compounds that bind to and inhibit the activity of cytochrome  $b_5$  in the physiological re-reduction of auto-oxidized hemoglobin (methemoglobin), e.g., compounds that interfere with the binding of hemoglobin and/or methemoglobin to cytochrome  $b_5$ . The invention further relates to pharmaceutical compositions comprising these compounds, and methods of using these pharmaceutical compositions to increase methemoglobin levels in the blood as a treatment for sickle cell disease.

Description of the Related Art

In the United States the occurrence of sickle cell disease, also known as sickle cell anemia, is relatively low, afflicting only about 70,000 Americans (Jones, C. P. 1995, *Pharmacy Today*, 1, 1-2), predominantly of African descent. Worldwide, however, sickle cell disease afflicts many millions of individuals (Bunn, H. F.; Forget, B. G. *Hemoglobin: Molecular, Genetic and Clinical Aspects*; W. B. Saunders Company: Philadelphia, 1986. 502-564). Despite the fact that the molecular mechanism of hemoglobin sickling is well understood (Eaton, W. A.; Hofrichter, J. 1990, *Adv. Protein Chem.*, 40, 63-279) and the role of sickling in the pathology of the disease is clear, a rationally based drug therapy is not available to patients despite many attempts to develop such a therapy over the last few decades (Orringer, E. P.; Casella, J. F.; Ataga, K. I.; Koshy, M.; Adams-Graves, P.; Luchtman-Jones, L.; Wun, T.; Watanabe, M.; Shafer, F.; Kutlar, A.; Abboud, M.; Steinberg, M.; Adler, B.; Swerdlow, P.; Terregino, C.; Saccente, S.; Files, B.; Ballas, S.; Brown, R.; Wojtowicz-Praga, S.; Grindel, J. M. 2001, *JAMA*, 286(17):2099; Abraham, D. J.; Perutz, M. F.; Phillips, S. E. 1983, *Proc. Natl. Acad. Sci. U S A*, 80, 324-328; Klotz, I. M.; Haney, D. N.; King, L. C. 1981 *Science*, 213, 724-731). Although agents that modify hemoglobin allosterism have been identified through site-directed drug design (Abraham, D. J.; Wireko, F. C.; Randad, R. S.; Poyart, C.; Kister, J.; Bohn, B.; Liard, J. F.; Kunert, M. P. 1992, *Biochemistry*, 31, 9141-9149), all attempts at hemoglobin-directed antisickling agents have been unsuccessful.

The clinical manifestations of sickle cell disease are highly variable (Bunn, H. F.; Forget, B. G. *Hemoglobin: Molecular, Genetic and Clinical Aspects*; W. B. Saunders Company: Philadelphia, 1986. 502-564; Serjeant, G. R. 2001, *Br. J. Haematol.*, 112, 3-18). In young children, the major concern is the incidence of stroke. Chronic hemolytic anemia, impairment of growth and higher susceptibility to infection are common systemic manifestations. Vaso-occlusive crises are the origin of the most severe symptoms, including stroke and cardiac involvement in cases of "chest syndrome". The incidence of three or more vaso-occlusive crises per year is highly correlated with mortality. With current treatment, life expectancy for sickle cell patients is 40 to 50 years.

Until recently, the only approach to the treatment of sickle cell disease was fluids and analgesics, such as morphine, administered upon the occurrence of vaso-occlusive crises. While transfusion therapy is commonly employed in pediatric cases where the risk of stroke is high, there are serious potential problems with long-term transfusion therapy (Serjeant, G. R. 2001, *Br. J. Haematol.*, 112, 3-18). The risk of stroke is predicted by transcranial doppler measurements of blood flow in the brain. Although transfusion therapy is effective in reducing vaso-occlusive crises, such as stroke, there are several drawbacks. Iron overload is a common side effect, and iron chelation therapy employing desferoxamine is a common adjuvant therapy. Long-term transfusion therapy also carries the risk of alloimmunogenic reactions. There is also a risk of disease transmission that has been minimized with recent advances in diagnostic procedures.

In 1995, hydroxyurea (Jones, C. P. 1995 Sickle Cell Therapy so Effective, Trials end early, *Pharmacy Today*, 1, 1-2; Charache, S.; Terrin, M. L.; Moore, R. D.; Dover, G. J.; Barton, F. B.; Eckert, S. V.; McMahon, R. P.; Bonds, D. R. 1995, *N. Engl. J. Med.*, 332, 317-1322; Rodgers, G. P. 1997 *Semin. Hematol.*, 34, 2-7.) became available for the treatment of sickle cell disease. Because it was already used in the treatment of certain leukemias, it was rapidly approved for clinical testing and passed through clinical trials faster than any other drug in recent times. In an extensive study involving nearly 300 sickle cell patients, the occurrence of vaso-occlusive crises was reduced to roughly 50% of that observed in the patients involved (Charache, S.; Terrin, M. L.; Moore, R. D.; Dover, G. J.; Barton, F. B.; Eckert, S. V.; McMahon, R. P.; Bonds, D. R. 1995, *N. Engl. J. Med.*, 332, 317-1322.). It is believed that hydroxyurea works by inducing expression of fetal hemoglobin, however, there are a number of controversies concerning the exact mechanism of action, given that benefits appear to begin before the development of significant levels of fetal hemoglobin (Bunn, H. F. 1999, *Blood*, 93, 1787-1789). Despite the advantages in the use of hydroxyurea, it is not without significant side effects. Hydroxyurea is myelosuppressive and thus patients must be monitored carefully (Rodgers, G. P. 1997 *Semin. Hematol.*, 34, 2-7). Hydroxyurea causes chromosomal fragmentation and is teratogenic and mutagenic but does not appear to be carcinogenic. Because of the mutagenicity and the potential carcinogenicity in the long-term, it is not approved for use in children. Rather, it is approved for use in patients who suffer more than three vaso-occlusive crises a year, a clinical pattern strongly correlated with mortality (Castro, O. 1999, *Br. J. Haematol.*, 107, 2-11).

Relatively recently, bone marrow transplantation has been found to be an effective cure for sickle cell disease (Serjeant, G. R. 2001, *Br. J. Haematol.*, 112, 3-18). This treatment was first discovered when a leukemia patient was given a bone marrow transplant and serendipitously was also cured of his sickle cell disease. Several hundred bone marrow transplants have been performed specifically for the purpose of treating sickle cell disease. This approach is only available to about 18% of sickle cell patients because of the requirement of an HLA matched sibling donor. The procedure is costly and carries significant risks. Mortality because of immune responses ranges from 10% to 15% and subsequent alloimmune responses can be problematic. Thus, although, bone marrow transplantation is a very promising true cure for the genetic disorder, it has significant limitations that prevent widespread use.

It is known that methemoglobin, oxyhemoglobin, and carbonmonoxyhemoglobin, effectively inhibit sickling in patients with sickle cell disease (Franklin, I. M.; Rosemeyer, M. A.; Huehns, E. R. 1983, *Br. J. Haematol.*, 54, 579-587). Furthermore, in individuals with congenital deficiencies in cytochrome b<sub>5</sub>, methemoglobin levels rise as high as 50% of total hemoglobin and derivatives in the blood, without any adverse clinical manifestations other than mild cyanosis. Clinical trials performed in the 1960's demonstrated the efficacy of methemoglobin in the suppression of vaso-occlusive crises but were limited by the rapid re-reduction of methemoglobin by cytochrome b<sub>5</sub> and hence required massive quantities of compounds such as sodium nitrite, benzocaine or para-aminopropiophenone to maintain sufficient levels of methemoglobin (Beutler, E. 1961, *J. Clin. Invest.*, 40, 56-68). Delivery of the appropriate quantities of these compounds was difficult and the prospect of good patient compliance with such a drug regimen was remote.

Thus, sickle cell disease is a serious problem for which no effective solution is available, and a potentially useful approach to the treatment of the disease would be to increase the amount of methemoglobin in patients having sickle cell disease.

Cytochrome b<sub>5</sub> is the terminal electron donor to methemoglobin in the physiological re-reduction of auto-oxidized hemoglobin (Abe, K.; Sugita, Y. 1979, *Eur. J. Biochem.*, 101, 423-428; Gerbaut, L. 1991 *Clin. Chem.*, 37, 2117-2120). Hemoglobin auto-oxidizes at approximately 3% per day. The inhibition of cytochrome b<sub>5</sub>'s action in the re-reduction of methemoglobin to hemoglobin would lead to an increase in methemoglobin levels and a treatment for sickle cell disease.

The structures for hemoglobin and its derivatives have been previously determined (Perutz, M. F. 1989 *TIBS*, 14, 42-44; Bolton, W.; Cox, J. M.; Perutz, M. F. 1968, *J. Mol. Biol.*, 33, 283-297) and cytochrome b<sub>5</sub> (Mathews, S.; Czerwinski, E. W.; Argos, P. *The X-*

*Ray Crystallographic Structure of Calf Liver Cytochrome b<sub>5</sub>*; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. VII, pp 107-147). Complete NMR assignments for the rat cytochrome b<sub>5</sub> were determined for both equilibrium forms (Guiles, R. D.; Basus, V. J.; Kuntz, I. D.; Waskell, L. 1992, *Biochemistry*, 31, 11365-11375; Guiles, R. D.; Basus, V. J.; Sarma, S.; Malpure, S.; Fox, K. M.; Kuntz, I. D.; Waskell, L. 1993, *Biochemistry*, 32, 8329-8340). In addition, extensive characterization of the structural, dynamic, and electrochemical properties of rat cytochrome b<sub>5</sub> have been performed (Dangi, B.; Sarma, S.; Yan, C.; Banville, D. L.; DiGate, R. J.; Guiles, R. D. 1998, *Biochemistry*, 37, 8289-830251-54; Dangi, B.; Blankman, J. I.; Miller, C. J.; Volkman, B. F.; Guiles, R. D. 1998, *J. Phys. Chem. B*, 102, 8201-8208; Sarma, S.; Banville, D.; DiGate, R. J.; Miller, C.; Guiles, R. D. 1997, *Biochemistry*, 36, 5658-5668; Cheng, J.; Terrettaz, S.; Blankman, J. I.; Miller, C. J.; Dangi, B.; Guiles, R. D. 1997, *Israel Journal of Chemistry*, 37, 259-266; Blankman, J. I.; Shahzad, N.; Dangi, B.; Miller, C. J.; Guiles, R. D. 2000, *Biochemistry*, 39, 14799-14805). Furthermore, theoretical studies of the hemoglobin and cytochrome b<sub>5</sub> complex have been performed (Poulos, T. L.; Mauk, A. G. 1983, *J. Biol. Chem.*, 258, 7369-7373).

#### Brief Description of the Invention

The present invention is directed to compounds that perform the function described above. The inventive compounds described herein comprise, e.g. parts R1, R2, and R3, wherein R1 is a moiety that binds to specific sites on cytochrome b<sub>5</sub> in a way that mimics hemoglobin binding to cytochrome b<sub>5</sub>; R2 is a flexible linker that covalently crosslinks R1 and R3; and R3 is a moiety that binds to specific sites on cytochrome b<sub>5</sub> in a way that mimics ATP binding to cytochrome b<sub>5</sub>. The inventive compounds further comprise, e.g., parts R1, R2, and R3, wherein: R1 is a moiety that inhibits hemoglobin binding to cytochrome b<sub>5</sub>; R2 is a flexible linker that covalently crosslinks R1 and R3; and R3 is a moiety that inhibits electron transfer to methemoglobin by cytochrome b<sub>5</sub>. The compounds of the invention further comprise, e.g., parts R1, R2, and R3, wherein R1 is a linear polyamine or a cyclic polyamine such as hexacyclen; R2 is a flexible linker which covalently crosslinks R1 and R3; and R3 is ATP or an ATP derivative.

The present invention is also directed to pharmaceutical compositions of the compounds described above and a method of administering the described compounds to achieve a therapeutic effect in a patient with sickle cell disease.

#### Brief Description of the Drawings

Figure 1 illustrates sections of contour plots of overlays of <sup>1</sup>H - <sup>15</sup>N HSQC spectra of cytochrome b<sub>5</sub> by itself and in complex with human methemoglobin. In Figure 1A, the concentration of cytochrome b<sub>5</sub> is 1 mM and the concentration of methemoglobin is 0.50 mM. In Figure 1B, the concentration of methemoglobin is 0.25 mM.

Figure 2 illustrates the heteronuclear correlation spectra (HSQC spectra) of a 2 mM solution of cytochrome b<sub>5</sub> by itself (black contours) and that of a solution containing 2 mM cytochrome b<sub>5</sub> and 4 mM hexacyclen (gray contours).

Figure 4 shows modification of hexacyclen to enable attachment of an R2 linker for use in crosslinking to the R3 moiety.

Figure 5 shows the thiolation of ADP that can then be linked to the derivatized polyamines.

Figure 6 shows linking of derivatized spermine to derivatized ADP.

Figure 7 shows attachment of the flexible spacer and covalent attachment of the two derivatized groups.

Figure 8 shows an HSQC overlay of a sample containing cytochrome b<sub>5</sub> and ATP (2 mM) and a sample of cytochrome b<sub>5</sub> alone.

Figure 9 shows a set of traces of the optical absorbance changes occurring at 577 nm for cytochrome b<sub>5</sub> and methemoglobin at 5  $\mu$ M concentrations. Various concentrations of buffer and of hexacyclen were examined. In trace A) the buffer concentration is 10 mM phosphate at pH 7.0. In trace B) the buffer concentration is 1 mM phosphate at pH 7.0. In trace C) the

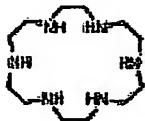
buffer concentration is 1.0 mM phosphate pH 7.0 and the concentration of hexacyclen is 100  $\mu$ M. In trace D and E) the concentration of phosphate is 1 mM pH 7.0 and the concentration of hexacyclen is 1 mM.

#### Detailed Description of the Invention

This invention relates, *e.g.*, to compounds having the structure R1-R2-R3 that bind to cytochrome  $b_5$  and inhibit the activity of cytochrome  $b_5$  in the reduction of methemoglobin to hemoglobin. Without wishing to be bound to any particular mechanism, it is proposed that these compounds prevent the binding of hemoglobin and/or methemoglobin to cytochrome  $b_5$  by binding at the methemoglobin/hemoglobin binding site on cytochrome  $b_5$  and preventing the electron transfer between methemoglobin/hemoglobin and cytochrome  $b_5$ . By binding to cytochrome  $b_5$  and preventing reduction of autoxidized hemoglobin, these compounds raise the level of methemoglobin in red blood cells and reduce the incidence of cell sickling. Thus, these compounds are useful for the treatment of sickle cell disease.

In one embodiment, the invention relates to a compound that consists of three parts, designated R1, R2 and R3 as described below. R1 and R3 bind to specific sites on the surface of cytochrome  $b_5$  as defined by shifts in  $^1\text{H}$ - $^{15}\text{N}$  heteronuclear correlation spectrum peaks defined below (Heteronuclear Single Quantum Coherence (HSQC) mapping: Mori, S.; Abeygunawardana, C.; Johnson, M. O. N.; van Zijl, P. C. M. 1995, *Journal of Magnetic Resonance, Series B*, 108, 94-98). R2 is a linker which covalently links R1 and R3.

In another embodiment, R1 is 1,4,7,10,13,16-hexaazacyclooctadecane (hexacyclen), the structure of which is as follows:

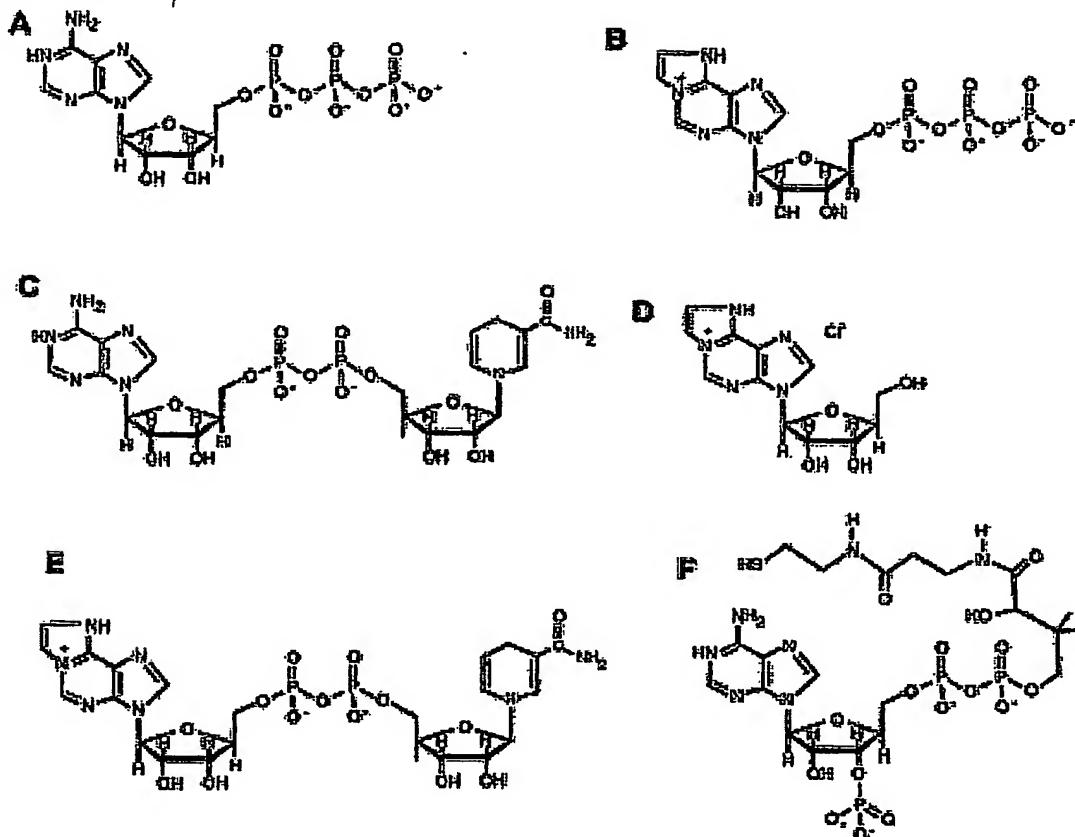


R1 can be hexacyclen or a derivative thereof that binds to cytochrome  $b_5$  in such a manner as to inhibit electron transfer from cytochrome  $b_5$  to methemoglobin. One can use an optical assay of electron transfer rate to identify such agents (see examples below). It is believed that agents which interfere with electron transfer between cytochrome  $b_5$  and methemoglobin also bind to one or more of the following cytochrome  $b_5$  residues: H26, E43, E44, E48, A54, D60, H80 and A88. Shifts in HSQC perturbation mapping (see examples below). (Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. 1996, *Science*, 274, 1531-4)

R2 is a linker between R1 and R3. It is preferable that the linker be flexible. A number of linkers, flexible and non-flexible, are known in the art field. By way of example only, R2 can be a polyglycine moiety containing between 1 and 3 glycines and derivatives thereof. As another example, R2 can be polyethylene glycol (PEG) and PEG-like moieties such as, but not limited to, polystyrene-PEG, [2-(2-aminoethoxy)ethoxy] acetic acid, allyloxycarbonyl-[2-(2-aminoethoxy)ethoxy] acetic acid, fluorenyl-methoxycarbonyl-[2-(2-aminoethoxy)ethoxy] acetic acid, ter-butyloxycarbonyl-[2-(2-aminoethoxy)ethoxy] acetic acid, benzyloxycarbonyl-[2-(2-aminoethoxy)ethoxy] acetic acid. Alternatively, R2 can be a straight chain or branched chain of carbon and hydrogen where the number of carbon atoms can be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, or more.

R3 is a moiety that binds to the site on cytochrome  $b_5$  distinct from the binding site of R1. It is preferable that R3 binds to cytochrome  $b_5$  in such a manner as to induce shifts in heteronuclear correlation peaks corresponding to one or more of following residues on cytochrome  $b_5$ : I24, L25, H26, and H27. Example of moieties which bind to cytochrome  $b_5$  and induce shifts in heteronuclear correlation peaks at one or more of those specified amino acids is ATP (adenosine 5'-triphosphate); 1,N6-ethenoadenosine 5' triphosphate;  $\beta$ -

nicotinamide adenine dinucleotide (NADH); 1,N6-ethenoadenosine hydrochloride; nicotinamide-1,6-ethenoadenosine; and coenzyme A. Other ATP analog exist and are well known in the art-field. ATP binds to cytochrome  $b_5$  with an affinity of 180  $\mu$ M. Below are the chemical structures of some R3 moieties:



A) Adenosine 5'-triphosphate (ATP); B) 1,N6-Ethenoadenosine 5' triphosphate; C)  $\beta$ -Nicotinamide adenine dinucleotide (NADH); D) 1,N6-ethenoadenosine hydrochloride; E) Nicotinamide-1,6-ethenoadenosine; F) Coenzyme A. All of the compounds are commercially available.

In another embodiment, the invention relates to a method for achieving a therapeutic effect in an animal having sickle cell disease and in need of treatment thereof, comprising administering an effective amount of the compound R1-R2-R3, where R1 and R3 bind to cytochrome  $b_5$  as discussed above and R2 is a linker as discussed above.

This invention also involves the usage of R1-R2-R3 to prevent the reduction of methemoglobin to hemoglobin such that methemoglobin accumulates in the blood. Such an accumulation of methemoglobin is useful for the prevention of sickling events in animals having sickle cell disease.

Cytochrome  $b_5$  plays an important role in reducing methemoglobin levels, as demonstrated experimentally and indicated in individuals with congenital deficiencies in cytochrome  $b_5$ , who have abnormally high levels of methemoglobin in their blood. Compounds of the invention can inhibit the activity of cytochrome  $b_5$  by, e.g., blocking the binding of methemoglobin to cytochrome  $b_5$ . Compounds of the invention can also achieve the inhibition of cytochrome  $b_5$  activity by, e.g., blocking electron transfer to methemoglobin. Compounds of the invention that are comprised of two moieties such that each moiety binds to different sites on cytochrome  $b_5$  have an affinity for cytochrome  $b_5$  that is greater than the

affinity of either moiety for its individual site. The compounds of the invention are thus highly effective at inhibiting cytochrome  $b_5$  activity and raising levels of methemoglobin in the blood.

The inventive compounds exhibit, e.g., therapeutic activity in raising levels of methemoglobin in the blood, and are effective in treating sickle cell disease by reducing the amount of cell sickling. In accordance with a preferred embodiment, the present invention includes methods of treating animals suffering from sickle cell disease.

The preferred aspects include pharmaceutical compositions comprising a compound of this invention having the structure R1-R2-R3 (wherein R1, R2, and R3 comprise the moieties described above) and a pharmaceutically acceptable carrier; a method of inhibiting the activity of cytochrome  $b_5$  in red blood cells by administering the pharmaceutical compositions to an animal; a method of increasing the levels of methemoglobin in red blood cells by administering the pharmaceutical compositions to an animal; and a method of treating sickle cell disease in a mammal, e.g., a human, by administering the pharmaceutical compositions to the mammal.

The present invention also relates to useful forms of the compounds as disclosed herein, such as pharmaceutically acceptable salts and prodrugs of all the compounds. The compounds of the invention can be administered alone or as an active ingredient of a formulation. Thus, the present invention also includes pharmaceutical compositions of compounds having the structure R1-R2-R3 (wherein R1, R2 and R3 comprise the moieties described above) containing, for example, one or more pharmaceutically acceptable carriers.

Numerous standard references are available that describe procedures for preparing various formulations suitable for administering the compounds according to the invention. Examples of potential formulations and preparations are contained, for example, in the *Handbook of Pharmaceutical Excipients*, American Pharmaceutical Association (current edition); *Pharmaceutical Dosage Forms: Tablets* (Lieberman, Lachman and Schwartz, editors) current edition, published by Marcel Dekker, Inc., as well as *Remington's Pharmaceutical Sciences* (Arthur Isol, editor), 1553-1593 (current edition).

In view of the high degree of selective inhibition of cytochrome  $b_5$  activity, the compounds of the present invention can be administered to any animal requiring inhibition of cytochrome  $b_5$  activity. Administration may be accomplished according to animal's needs, for example, by intravenous injection. Various solid oral dosage forms can be used for administering compounds of the invention including such solid forms as tablets, gelcaps, capsules, caplets, granules, lozenges and bulk powders. The compounds of the present invention can be administered alone or combined with various pharmaceutically acceptable carriers, diluents (such as sucrose, mannitol, lactose, starches) and excipients known in the art, including but not limited to suspending agents, solubilizers, buffering agents, binders, disintegrants, preservatives, colorants, flavorants, lubricants and the like. Time-release capsules, tablets and gels are also advantageous in administering the compounds of the present invention.

Various liquid oral dosage forms can also be used for administering compounds of the inventions, including aqueous and non-aqueous solutions, emulsions, suspensions, syrups, and elixirs. Such dosage forms can also contain suitable inert diluents known in the art such as water and suitable excipients known in the art such as preservatives, wetting agents, sweeteners, flavorants, as well as agents for emulsifying and/or suspending the compounds of the invention. The compounds of the present invention may be injected, for example, intravenously, in the form of an isotonic sterile solution. Other preparations are also possible.

The compounds can be administered as the sole active agent or in combination with other pharmaceutical agents, such as other agents that raise levels of hemoglobin variants in the red blood cells in order to prevent cell sickling in animals with sickle cell disease.

The dosages of the compounds of the present invention depend upon a variety of factors including the severity of the symptoms, the age, sex and physical condition of the animal, the route of administration, the frequency of the dosage interval, the particular compound utilized, the efficacy, toxicology profile, pharmacokinetic profile of the compound, and the presence of any deleterious side-effects, among other considerations.

By "effective dose" or "therapeutically effective dose" is meant herein, in reference to the treatment of sickle cell disease, an amount sufficient to bring about one or more of the following results: increase the level of methemoglobin in the blood above about 3%; reduce the

level of pain related to sickle cell disease; or reduce the incidence of sickle cell crises. The compounds of the invention can be administered at dosage levels and in a manner customary for ticlopidine hydrochloride, or other drugs used to treat sickle cell disease. For example, ticlopidine hydrochloride is administered at 250 mg bi-daily (see PDR).

In carrying out the procedures of the present invention it is of course to be understood that reference to particular buffers, media, reagents, cells, culture conditions and the like are not intended to be limiting, but are to be read so as to include all related materials that one of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is often possible to substitute one buffer system or culture medium for another and still achieve similar, if not identical, results. Those of skill in the art will have sufficient knowledge of such systems and methodologies so as to be able, without undue experimentation, to make such substitutions as will optimally serve their purposes in using the methods and procedures disclosed herein.

#### Example 1

The interaction of cytochrome  $b_5$  with hemoglobin is explored using HSQC perturbation mapping. Figure 1 contains sections of contour plots of overlays of  $^1\text{H} - ^{15}\text{N}$  HSQC spectra of cytochrome  $b_5$  by itself and in complex with human methemoglobin. Significant shifts in the positions of a number of residues of cytochrome  $b_5$  are observed. In Figure 1A, the concentration of cytochrome  $b_5$  is 1 mM and the concentration of methemoglobin is 0.50 mM. The black contours are of a heteronuclear correlation spectrum of cytochrome  $b_5$  by itself while the gray contours are of a sample containing both cytochrome  $b_5$  and methemoglobin. In Figure 1B, the concentration of methemoglobin is 0.25 mM. The pH in all cases was 6.4 and the temperature was 25 °C. A number of residues that shift significantly on complex formation are labeled (e.g. most notably H26, E43, E44, A54, H80 and A88). A number of peaks that do not shift significantly on complex formation are also labeled (i.e. K5 and Y30). Heteronuclear correlation spectra were recorded using the fast HSQC sequence (Mori, S.; Abeygunawardana, C.; Johnson, M. O. N.; van Zijl, P. C. M. 1995, *J. Mag. Reson. B*, 108, 94-98). The shifts in heteronuclear correlation peaks observed on complex formation are consistent at least in part with the theoretical model of the complex between cytochrome  $b_5$  and methemoglobin. Note that shifts in peaks associated with residues E43, E44 and probably A54 via a relayed effect in helix V of cytochrome  $b_5$  are consistent with the theoretical model. Compounds which interact with cytochrome  $b_5$  at one or more of the following amino acids of cytochrome  $b_5$  may interfere with or prevent the binding of methemoglobin to cytochrome  $b_5$ , the amino acids being H26, E43, E44, A54, H80 and A88.

#### Example 2

Hexacyclen (1,4,7,10,13,16-hexaazacyclooctadecane) (Richman, J. E.; Atkins, T. J. 1974, *J. Am. Chem. Soc.*, 96, 2268-2269) binds to cytochrome  $b_5$  such the HSQC spectra of cytochrome  $b_5$ -hexacyclen is similar to the HSQC spectra of cytochrome  $b_5$ -hemoglobin. The concentration dependence of hexacyclen-induced heteronuclear correlation peak shifts indicates a dissociation constant of roughly 2 mM. Figure 2 illustrates the heteronuclear correlation spectra (HSQC spectra) of a 2 mM solution of cytochrome  $b_5$  by itself (black contours) and that of a solution containing 2 mM cytochrome  $b_5$  and 4 mM hexacyclen (gray contours). The inset in the upper left hand corner of the figure contains a plot of the hexacyclen dependence of the shifts in the peak to peak separation of aspartate 60 (D60) at concentrations of hexacyclen ranging from 0.5 to 8 mM. The inset at the upper right is a model for the interaction of hexacyclen based on the shifts observed in the HSQC perturbation study. Solutions were buffered to a pH of 7.0 with 1 mM phosphate buffer and the spectra were recorded at 40 °C. The inset in the upper left hand corner of the figure contains a plot of the hexacyclen dependence of the shifts in the peak to peak separation of aspartate 60 (D60) at concentrations of hexacyclen ranging from 0.5 to 8 mM. The inset at the upper right is a model for the interaction of hexacyclen based on the shifts observed in the HSQC perturbation study.

#### Example 3

Work was performed on cytochrome  $b_5$  in an attempt to find compounds which inhibit or increase cytochrome  $b_5$ 's properties, such as the reduction potential and binding affinity to cytochrome  $b_5$ 's electron transfer partners by relayed effects (Rivera, M.; Wells, M. A.; Walker, F. A. 1994, *Biochemistry*, 33, 2161-2170; Vergères, G.; Waskell, L. 1995, *Biochemie*, 77, 604-620; Reid, L. S.; Gray, H. B.; Dalvit, C.; Wright, P. E.; Saltman, P. 1987, *Biochem.*, 26, 7102-7107). ATP binds to cytochrome  $b_5$  with an affinity of 180  $\mu$ M (Reid, L. S.; Gray, H. B.; Dalvit, C.; Wright, P. E.; Saltman, P. 1987, *Biochem.*, 26, 7102-7107). ATP binds to a hydrophobic domain between the four helix bundle that binds the heme and the  $\beta$ -turn in the  $\beta$ -sheet region on cytochrome  $b_5$ , this binding site being distinct from the binding site of hexacyclen.

#### Example 4

#### Example 5

Schemes for the derivatization of hexacyclen (R1) have been developed using minor modifications of the Richman-Atkins synthesis (Richman, J. E.; Atkins, T. J. 1974, *J. Am. Chem. Soc.*, 96, 2268-2269). The R group in the scheme shown in Figure 4 was a tosyl group in the original synthesis, but has been replaced with a carbamyl group in this modification, which can be selectively removed using L-selectride (Coop, A.; Rice, K. C. 1998, *Tet. Lett.*, 39, 8933-8934), lithium tri-sec-butylborohydride (Aldrich), under mild conditions which will not remove the Ts group. Figure 4 shows a modified Richman-Atkins synthesis of hexacyclen to enable attachment of a derivatizable R group for use in crosslinking to the R3 moiety. A scheme utilizing this modified hexacyclen in the preparation of a polypeptide that is used to link the thiolated ADP shown in Figure 5 using a bifunctional crosslinking agent is shown in Figure 7.

#### Example 6

In this scheme to link hexacyclen (R1) with an ADP derivative (R3), described by Hermanson (Hermanson, G. T. *Bioconjugate Techniques*; Academic Press, 1995.785 pp649-655), a water soluble carbodiamide is used to activate the terminal phosphate which can then be thiolated with  $\beta$ -mercaptoethylamine via cystamine following reductive cleavage with Cleland's reagent as shown in Figure 5. As shown in Figure 5, a water soluble carbodiamide is used to activate the terminal phosphate that can then be thiolated with  $\beta$ -mercaptoethylamine via cystamine following reductive cleavage with Cleland's reagent.

#### Example 7

In this scheme to link hexacyclen (R1) with an ADP derivative (R3), a polypeptide containing a C-terminal lysine and N-termination with the derivatized spermine was prepared using standard solid phase methods (Grant, G. A. *Synthetic Peptides: A User's Guide*; W. H. Freeman and Company: New York, 1992, 382). This polypeptide is then crosslinked to the derivatized ADP shown in Figure 4 using the bifunctional crosslinker BMPS (N-( $\beta$ -maleimido-propyloxy)succinimide) (McKenzie, J. A.; Raison, R. I.; Rivett, E. E. 1988, *J. Protein Chem.*, 7, 581-592) as shown in Figure 6.

#### Example 8

A similar scheme for linking a polypeptide with an N-terminal hexacyclen derivative to the thiolated ADP is shown in Figure 7. In this scheme a polypeptide with a C-terminal lysine and a variable number of glycines is prepared using standard solid phase synthesis techniques. The N-terminal group here is the derivatized hexacyclen.

#### Example 10

The binding of the R1 and R3 moieties to cytochrome  $b_5$  is characterized using HSQC perturbation mapping experiments. Figure 2 contains an overlay of heteronuclear correlation

spectra (HSQC spectra) of a 2 mM solution of cytochrome  $b_5$  by itself (black contours) and that of a solution containing 2 mM cytochrome  $b_5$  and 4 mM hexacyclen (gray contours), illustrating shifts due to the binding of hexacyclen to  $^{15}\text{N}$ -labeled cytochrome  $b_5$ . Figure 2 can be compared with Figure 8, which contains an HSQC overlay of a control on that of a sample containing cytochrome  $b_5$  and ATP. Solutions were buffered to a pH of 7.0 with 1 mM phosphate buffer and the spectra were recorded at 40 °C. Figure 8 contains an HSQC overlay of a control on that of a sample containing cytochrome  $b_5$  and ATP at 2 mM concentration. Although there is some overlap in peaks affected by the binding of ATP with that seen with the binding of hexacyclen, some of these effects are probably relayed.

#### Example 11

In addition to the site interaction studies using NMR, functional assays of inhibition of electron transfer have been performed using manual mixing experiments, similar to those described by Sugita (Abe, K.; Sugita, Y. 1979, *Eur. J. Biochem.* 101, 423 - 428). The electron transfer reactions were monitored by observing absorbance changes at 577 nm similar to experiments performed by McLendon's group (Qiao, T.; Simmons, J.; Horn, D. A.; Chandler, R.; McLendon, G. 1993, 97, 13089-13091). Figure 9 contains a set of traces of the optical absorbance changes occurring at 577 nm for cytochrome  $b_5$  and methemoglobin at 5  $\mu\text{M}$  concentrations with 1 mM phosphate buffer at pH 7.0. Various concentrations of hexacyclen were examined ranging from 100  $\mu\text{M}$  to 1 mM. The concentration of phosphate buffer was also examined in order to assess the effect of ionic strength on the rate of the reaction. In all cases the concentration of cytochrome  $b_5$  and methemoglobin is 5  $\mu\text{M}$  and the temperature was maintained at 37°C. For trace A, the buffer concentration is 10 mM phosphate at pH 7.0. For trace B, the buffer concentration is 1 mM phosphate at pH 7.0. For trace C, the buffer concentration is 1.0 mM phosphate, pH 7.0 and the concentration of hexacyclen is 100 mM. For trace D and trace E, the concentration of phosphate is 1 mM, pH 7.0 and the concentration of hexacyclen is 1 mM.

While the disclosure above describes the invention in detail and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Statement of Invention

1. A compound of the general formula R1-R2-R3, wherein R1 comprises a moiety that binds to specific sites on cytochrome b<sub>5</sub> such that said binding mimics hemoglobin binding to cytochrome b<sub>5</sub>; R3 comprises a moiety that binds to specific sites on cytochrome b<sub>5</sub> such that said binding mimics ATP binding to cytochrome b<sub>5</sub>; and R2 comprises a linker moiety which links R1 and R3.
2. The compound of claim 1, wherein R1 is a linear polyamine.
3. The compound of claim 1, wherein R1 is a cyclic polyamine.
4. The compound of claim 1, wherein R3 is an ATP derivative.
5. The compound of claim 1, wherein R1 is hexacyclen.
6. The compound of claim 1, wherein R3 is ATP.
7. A pharmaceutical composition, comprising the compound of claim 1 or a pharmaceutically acceptable salt thereof.
8. A method of achieving a therapeutic effect in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 1.
9. A method of reducing the incidence of red blood cell sickling in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 1.
10. A compound comprising parts R1, R2, and R3, wherein R1 comprises a moiety that inhibits hemoglobin binding to cytochrome b<sub>5</sub>; R2 comprises a flexible linker which covalently crosslinks R1 and R3; R3 comprises a moiety that inhibits electron transfer to methemoglobin by cytochrome b<sub>5</sub>.
11. .
12. .
13. The compound of claim 10, wherein R3 is an ATP derivative.
14. The compound of claim 10, wherein R1 is hexacyclen.
15. The compound of claim 10, wherein R3 is ATP.
16. A pharmaceutical composition, comprising the compound of claim 10 or a pharmaceutically acceptable salt thereof.
17. A method of achieving a therapeutic effect in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 10.
18. A method of reducing the incidence of red blood cell sickling in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 10.
19. A compound of the general formula R1-R2-R3, wherein

R1 comprises a moiety that binds to cytochrome b<sub>5</sub> at one or more amino acids selected from the group consisting of H26, E43, E44, E48, A54, D60, H80 and A88;

R3 comprises a moiety that binds to cytochrome b<sub>5</sub> distinct from said binding of said R1 to cytochrome b<sub>5</sub>; and

R2 comprises a linker moiety which links R1 and R3.

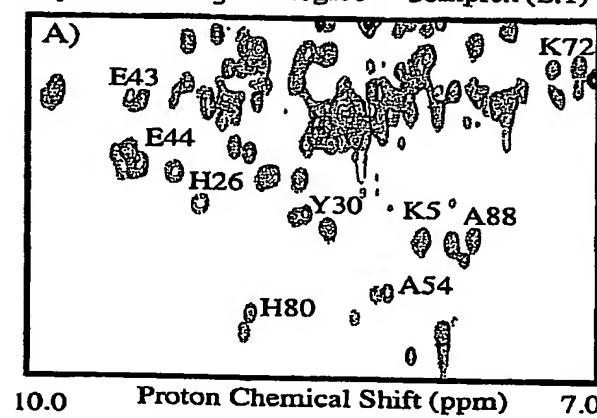
20. A pharmaceutical composition, comprising the compound of claim 19 or a pharmaceutically acceptable salt thereof.
21. A method of achieving a therapeutic effect in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 19.
22. A method of reducing the incidence of red blood cell sickling in an animal with sickle cell disease, comprising administering an effective amount of the compound of claim 19.
23. The method of claim 8, claim 9, claim 17, claim 18, claim 21, or claim 22, wherein said animal is human.

Abstract

Compounds have been designed to inhibit the action of cytochrome b<sub>5</sub> in the physiological re-reduction of auto-oxidized hemoglobin (methemoglobin), for the purpose of increasing methemoglobin levels in the blood of patients as a treatment for sickle cell disease. Sickle cell disease afflicts millions worldwide, causing morbidity and mortality, and current drug therapies are inadequate and have serious side effects. Bone marrow transplant is a cure currently available only to a fraction of patients, also with serious side effects. This invention consists of variants of endogenous, non-toxic compounds. Administration of the compounds mimics congenital deficiencies in cytochrome b<sub>5</sub>, in which methemoglobin levels rise as high as 50% of total hemoglobin and derivatives in the blood, without adverse clinical manifestations. Methemoglobin inhibits red cell sickling and high levels of methemoglobin in the blood induced by the compounds of this invention prevent the symptoms of sickle cell disease.

50443733.013003

Cytochrome  $b_5$  Hemoglot Complex (2:1)



Cytochrome  $b_5$  He: globin Complex (4:1)

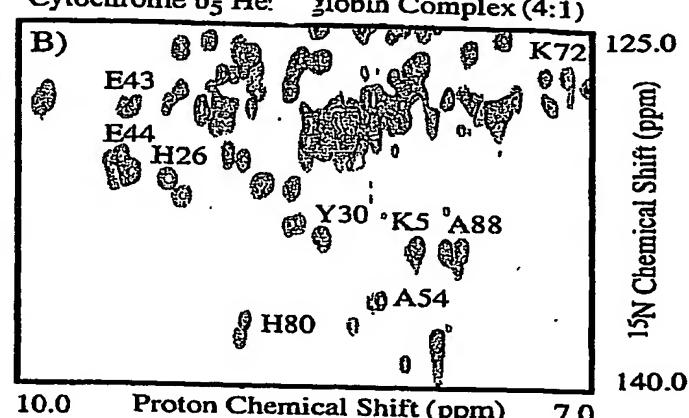


Figure 1

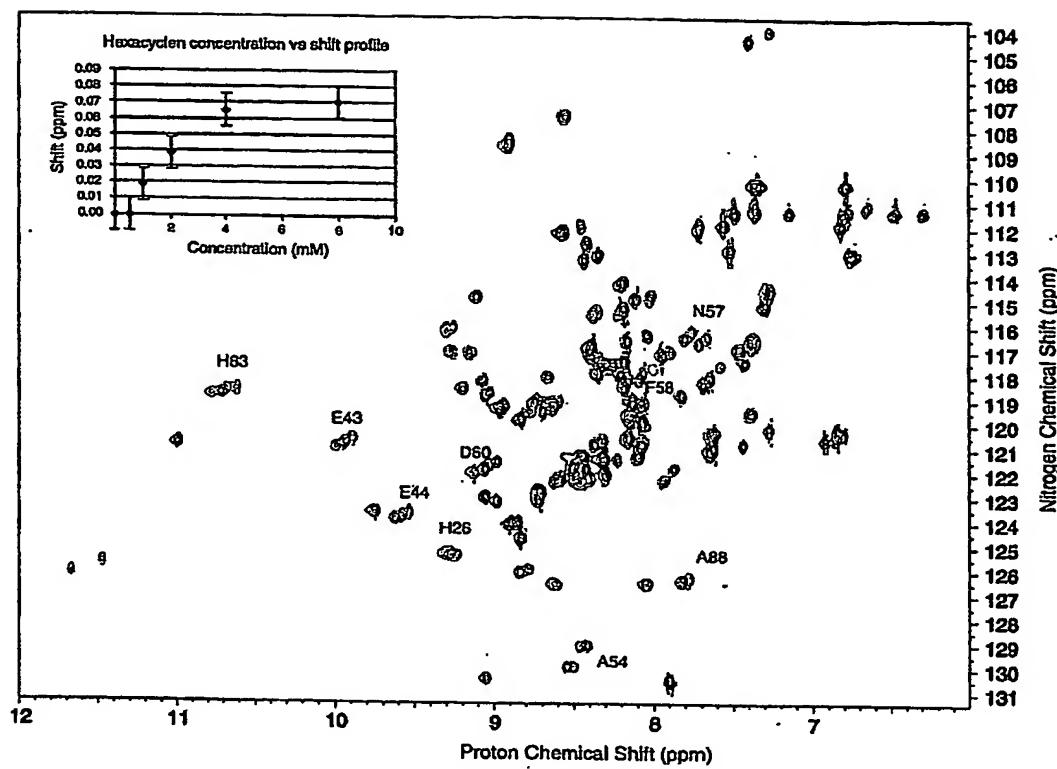


Figure 2

60443783 · 013003

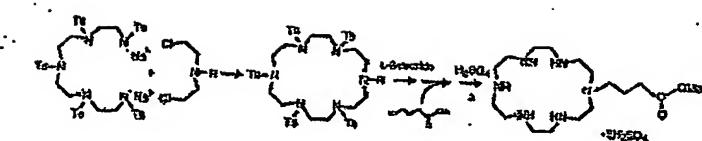


Figure 4

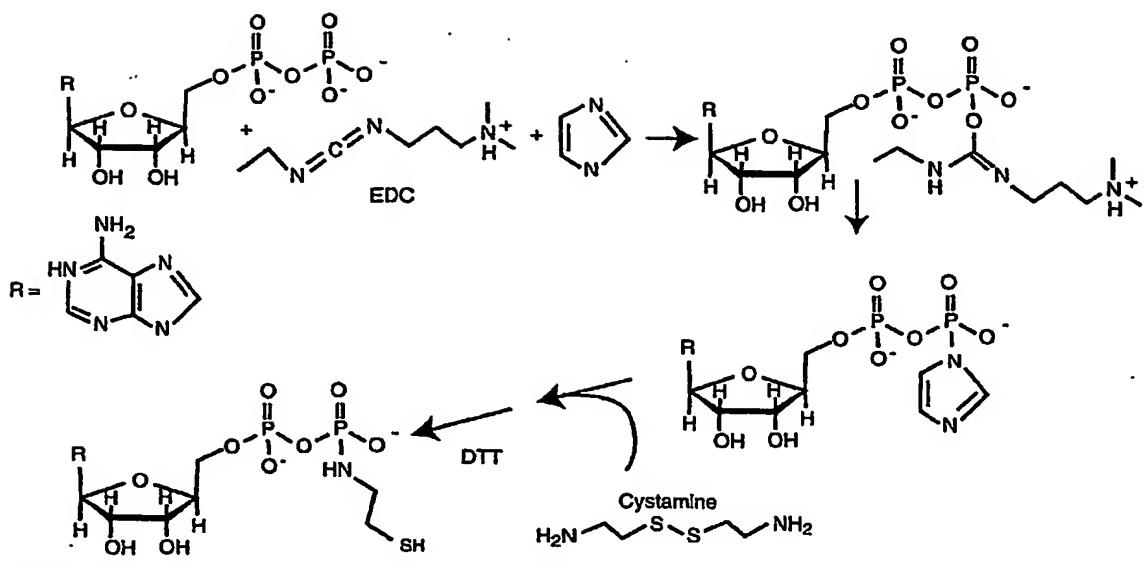


Figure 5

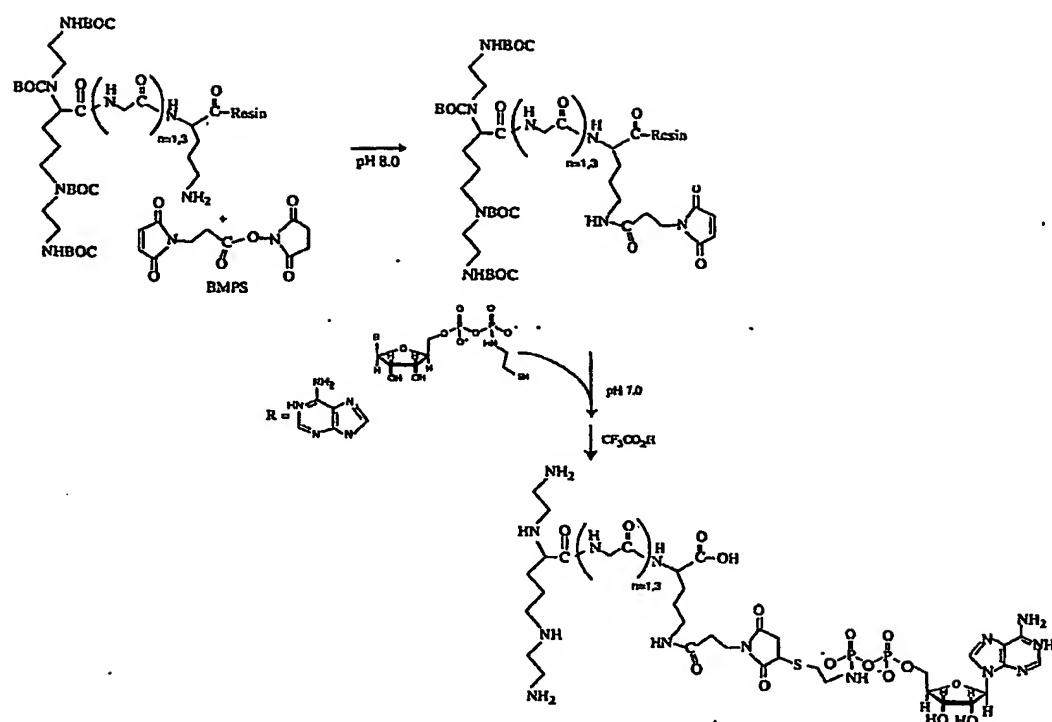


Figure 6

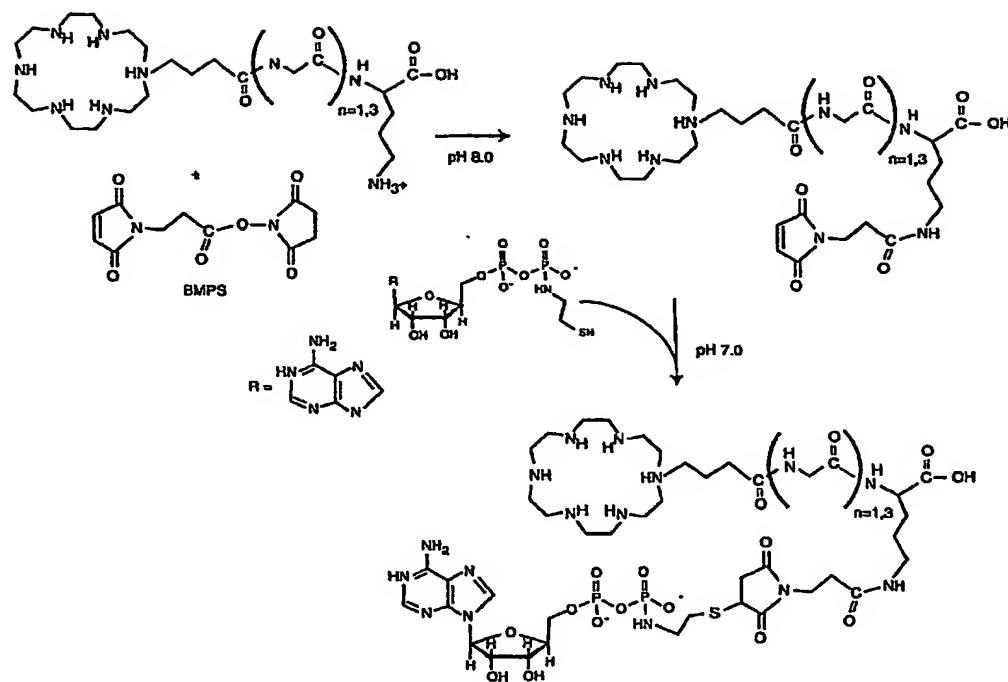


Figure 7

60443783 .013003

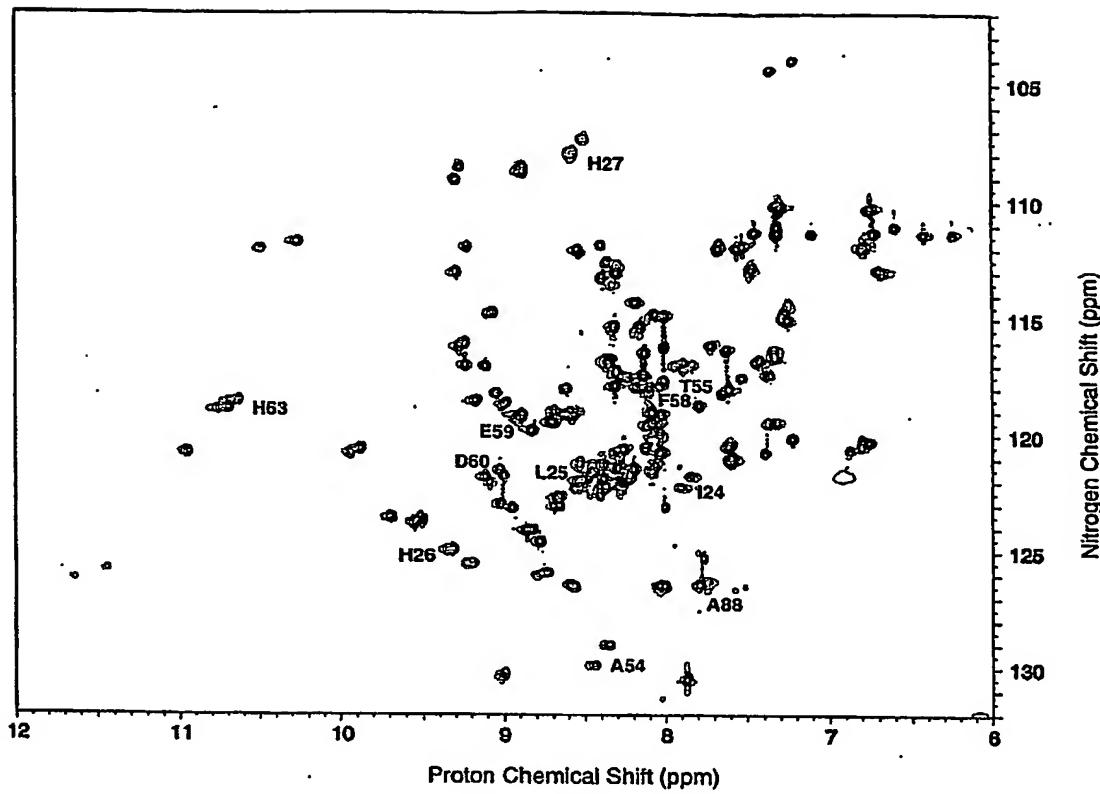


Figure 8

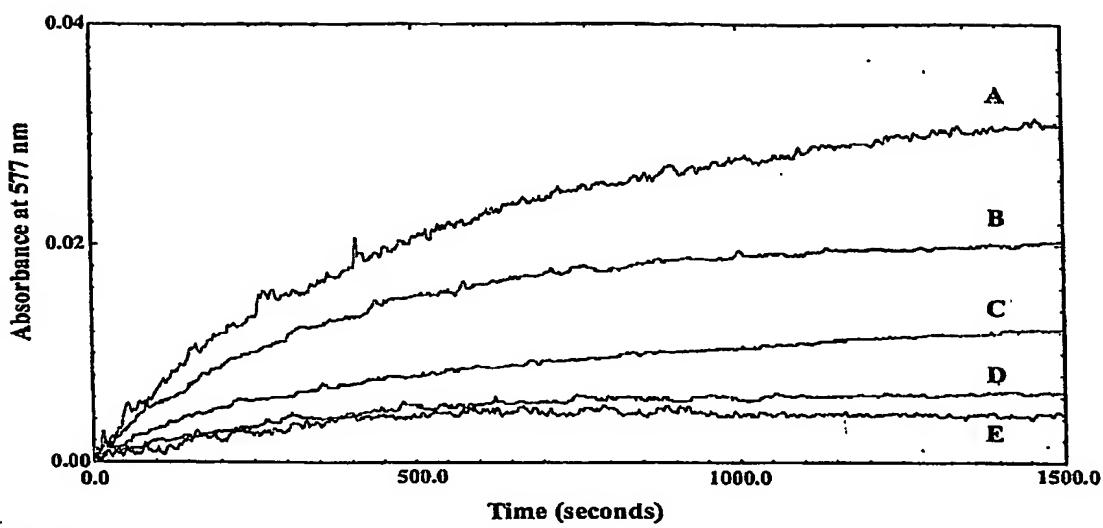


Figure 9

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**